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Abstract

The human embryonic stem cell line RCe016-A (RC-12) was derived under quality assured compliance with UK regulation, EU Directives and International guidance for tissue procurement, processing and storage according to Good Manufacturing Practice (GMP) standards. The cell line was derived from a cryopreserved blastocyst stage embryo voluntarily donated as surplus to fertility requirements following informed consent. RCe016-A (RC-12) shows normal pluripotency marker expression and differentiation to three germ layers in vitro. Karyology revealed a mixed male karyotype at early passage (P15), which resolved as normal 46XY by passage 33. Microsatellite PCR identity, HLA and blood group typing data is available.

Resource table

Name of stem cell construct	RCe016-A
Alternative name	RC-12, RC12
Institution	Roslin Cells Ltd.
Person who created resource	B.J. Tye, K. Bruce, P. Dand, G. Russell, D.M. Collins, A. Greenshields, K. McDonald, H. Bradburn, A. Laurie
Contact person and email	Paul.desousa@roslincells.com; Paul.desousa@ed.ac.uk Janet.downie@roslincells.com Aidan.courtney@roslincells.com Malcolm.bateman@roslinfoundation.com
Date archived/stock	07 September 2010 (seed bank)

date	
Type of resource	Biological reagent: cell line
Sub-type	hESC, clinical grade
Origin	Blastocyst with ICM and Trophoblast
Key transcription factors	Oct4 (confirmed by flow cytometry),
Authentication	See Quality Control Certificate of Analysis (Fig. 1)
Link to related literature (direct URL links and full references)	N/A
Information in public databases	http://hpscreg.eu/cell-line/RCe016-A
Ethics	Informed consent obtained. Scotland A Research Ethics committee approval obtained (07/MRE00/56). Conducted under the UK Human Fertilisation and Embryology Authority licence no R0136 to centre 0202 and UK Human Tissue Authority (HTA) licensing number 22631.

Resource details

RCe016-A (RC-12) was derived from a frozen and thawed blastocyst stage embryo that was surplus to fertility treatment requirement. Human embryonic stem cell (hESC) isolation, expansion and qualification was performed in a facilities whose specification, operation and monitoring complied with GMP standards enabling; i) a fully traceable procurement procedure with informed ethical consent which includes provision for commercial use, ii) detailed medical history and blood borne virus (BBV) screening of donors, and iii) compilation of a cell line history providing details on hESC manufacturing process and quality control testing regime.

Human ESC culture and processing was performed in a grade A tissue culture cabinet in a grade B clean room environment monitored for particulate and microbiological contamination during cell processing in accordance with Rules and Guidance for Pharmaceutical Manufacturers and Distributors - The Orange Guide, compiled by the UK Medicines Healthcare Products Regulatory Authority (Go to: https://www.gov.uk/guidance/good-manufacturing-practice-and-good-

distribution-practice). Accordingly, the facility was operating under a mature Quality Management System, compliant with ISO9001:2008 standards. HESC derivation was performed under licensure from the UK HFEA (R0136 to centre 0202) and HTA (Licensing Number 22631).

HESC derivation involved whole embryo outgrowth on mitotically inactivated human dermal fibroblast (HDF) feeder cells. HDFs were derived and manufactured according to GMP and had been approved for clinical use by the Food and Drug Administration, USA. During derivation on HDFs, hESCs were grown in a xeno-free cell therapy grade media (XF KODMEM) supplemented with xeno-free human recombinant bFGF. HESCs were subsequently expanded in a GMP grade serum-free medium (StemPro hESC Serum Free Medium,) on a xeno-free matrix (CellStart). The former contained bovine serum albumin (BSA) from a Transmissible Spongioform Encephalopathy (TSE)-free country of origin. The cell line was cryopreserved in a GMP compliant cryopreservation solution (CryoStor CS10,).

By flow cytometry, RCe016-A (RC-12) expressed the pluripotency makers Oct-4, Tra-1-60 and SSEA-4 (88.8%, 85.8% and 95.0%, respectively), whereas low expression of the differentiation marker SSEA-1 (0.1%) was observed (Fig. 1, Fig. 2). Differentiation to the three germ layers, endoderm, ectoderm and mesoderm, was demonstrated using embryoid body formation in vitro, and expression of the germ layer markers α-fetoprotein, β-tubulin and muscle actin was observed (Fig. 3).

A microsatellite PCR profile has been obtained for the cell line, and HLA Class I and II typing is available (Table 1). Blood group genotyping gave the blood group AO_1 (Table 1).

Verification and authentication

The cell line was analysed for genome stability by G-banding and showed a mixed male genotype at passage 15 (Certificate of Analysis, Fig. 1). A normal 46XY genotype was present in 18 of 30 cells analysed, but a subpopulation (12 of 30 cells) exhibited trisomy 12 (47XY, +12). By passage 33, the cell line had stabilised on a normal male 46XY karyotype (Fig. 4). The cell line is free from mycoplasma contamination as determined by RT-qPCR.

Materials and methods

Ethics

Derivation of hESC from surplus to requirement and failed to fertilise/develop embryos was approved by The Scotland A Research Ethics Committee and local ethics board at participating fertility clinics and conducted under licence no R0136 from the UK HFEA with informed donor consent. The processing and storage of hESC cells for human application was conducted under licence number 22631 from the UK Human Tissue Authority.

Cell culture

Frozen embryos were thawed using Vitrified Embryo Safety Thawing Pack (Kitazato/Dibimed, Valencia, Spain) according to manufacturers instruction and were cultured in system SAGE Quinn's Advanced Blastocyst medium (Rochford Medical, Coventry) after day 3 of development. Embryos were cultured at 36.5 - 37.5°C, 5.0 ± 0.5% CO_2, 5.0 ± 0.5% O_2 in drops under paraffin oil (Rochford Medical) and transferred to fresh medium at least every 2-3 days.

By Day 8 of development, embryos were placed in derivation conditions consisting of mitotically inactivated GMP grade neonatal human dermal fibroblasts (HDFs) (Forticell Biosciences, NJ, USA) on tissue culture plastic in XF KODMEM medium (Knockout-DMEM, 15% KOSR-XF, 2 mM L-glutamine, 1% MEM Non essential amino acids, 2% XF Growth Factor Cocktail, 0.1 mM β-mercaptoethanol (ThermoFisher Scientific, Paisley, UK) supplemented with 80 ng/ml human bFGF (ThermoFisher Scientific). When available, cell therapy system quality reagents were used. Assisted hatching was performed by removing the zona pellucidae mechanically using Swemed cutting tools (Vitrolife, Göteborg, Sweden).

HDF cells were cultured in DMEM (Lonza, Slough, UK), 10% Pharma grade FCS (GE Healthcare (PAA), Buckinghamshire, UK) and 2 mM L-glutamine (ThermoFisher Scientific). HDF were mitotically inactivated using gamma irradiation at 50Gy using a Gammacell Elite 1000 machine. For use as a feeder layer, irradiated HDFs were plated at 50000 cells/cm^2 in XF KODMEM medium supplemented with 80 ng/ml human bFGF (ThermoFisher Scientific). Cells were cultured at 36.5 - 37.5°C, 5.0 ± 0.5% CO_2, 5.0 ± 0.5% O_2 and 50% medium exchanged 6 days a week.

The established cell line was expanded and banked using CellStart matrix and Stempro hESC Serum Free Medium (cell therapy system quality reagents, ThermoFisher Scientific). Passaging was performed mechanically using an EZ passage tool (ThermoFisher Scientific). hESC lines were expanded to 25-30 wells of a 6-well plate and cryopreserved in 0.5-1 ml Cryostor CS10 (Biolife Solution, Washington, USA) using an EF600-107 controlled rate freezer (Grant Instruments, Cambridge, UK) before being stored in a -150°C freezer (Panasonic Biomedical, Loughborough, UK).

Mycoplasma

In process mycoplasma detection was performed using Applied Biosystems PrepSEQ™ Mycoplasma Nucleic Acid Extraction Kit and MicroSEQ™ Mycoplasma Real-Time PCR Detection Kit (ThermoFisher Scientific (Applied Biosystems)) according to the manufacturer's instruction. Eurpoean pharmacopoeia (EP) mycoplasma testing was carried out by Moredun Scientific Ltd. (Edinburgh, UK), under a quality and technical agreement.

Endotoxin

Endotoxin levels were determined using the Kinetic-QCL assay (Lonza) and an incubating plate reader (BioTek ELx808) according to the manufacturer's instructions. Briefly, an unknown sample was compared with a standard curve of known levels of control endotoxin. An assay was deemed valid if the coefficient of correlation, $r \geq 0.980$ and the CV (%) for the standard curve was ≤10%, and the reaction time of the negative control was greater than the reaction time of the lowest standard on the standard curve.

Flow cytometry

Pluripotency was determined using the Human and Mouse Pluripotent Stem Cell Analysis kit (BD, Oxford, UK). Oct 3/4 and SSEA-4 were included as pluripotency markers, and SSEA-1 as a differentiation marker. FITC conjugated Tra-1-60 (BD) was used as an additional pluripotency marker. Fixed and permeabilised cells were analysed using a FACS Aria flow cytometer (BD) or a Guava easyCyte flow cytometer (Millipore, Watford, UK). Percentage expression of each marker was compared to isotype control or unstained cells.

Viability

Viability was determined using the Guava ViaCount assay. Briefly, the Guava Viacount reagent (Millipore) containing a nuclear and a viability dye, was mixed with a single cell suspension, incubated for 5 minutes and analysed using the Guava easyCyte flow cytometer (Millipore). Total cell count, viable cell count and percentage viable cells was obtained.

In vitro differentiation

hESCs were pre-treated for 1 h with 10 μM ROCK inhibitor in Stempro hESC SFM (ThermoFisher Scientific) and embryoid bodies (EBs) generated in ultra low attachment plates (Corning) for 7 days before being transferred into EB medium (20% FBS (GE Healthcare (PAA)), 80% KO-DMEM 1 mM L-glutamine, 3.5 μM β-mercaptoethanol, 1 % nonessential amino acids (all ThermoFisher Scientific)), on glass slide tissue culture chambers (Nunc, ThermoFisher Scientific) coated with 0.2% gelatin (Sigma Aldrich, Dorset, UK) at 0.1 ml/cm^2 for 14 days.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (ThermoFisher Scientific (Alfa Aesar)), permeabilised using 100% ethanol (ThermoFisher Scientific) and stained with AFP (1:500; Sigma), β-tubulin III (1:1000; Sigma) and muscle-specific actin (1:50; DAKO, Glostrup, Denmark) and secondary antibody anti mouse IgG-AlexaFluor 488 (1:200; Sigma). Images were acquired using a Zeiss S100 Axiovert fluorescence microscope or Nikon eC1 confocal microscope

Genomic analysis and outsourced assays

All outsourced assays were carried out under a Quality and Technical Agreement. DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Manchester, UK) according to manufacturer's recommendations and provided in recommended quantities to the service providers.

Microsatellite PCR, or Short Tandem Repeat analysis, was used to determine cell line identity and was carried out by Public Health England. A profile was obtained for the following core alleles: vWA, D16S539, Amelogenin, THO1, CSF1PO, D5S818, D75820, D135317 and TPOX.

Human Leukocyte Antigen (HLA) tissue typing was carried out by the Scottish National Blood Transfusion Service.

Blood group genotyping was carried out by the Molecular Diagnostics laboratory at NHSBT.

Karyotype analysis was carried out by The Doctors Laboratory (London, UK) or the Western General Cytogenetics Laboratory (Edinburgh, UK). Live cells at 60-70% confluency were shipped in warm containers, fixed and analysed by standard G-banding analysis. For clinical grade lines, 30 spreads were analysed.

Viral screening for cytomegalovirus (CMV), Human T-cell lymphotropic virus (HTLV), Human immunodeficiency virus (HIV)-1, Hepatitis C virus (HCV), Hepatitis B virus (HBV), Epstein-Barr virus (EBV) and syphilis was carried out by The Doctors Laboratory.

Figures and tables

ROSLIN CELLS

Quality Control Test Certificate

Sample Point 2 Test Results

Certificate Number:	QCC-11-012	**Version:**	3
Grade:	CLINICAL		
Sample ID:	RC-12 P8B CS		

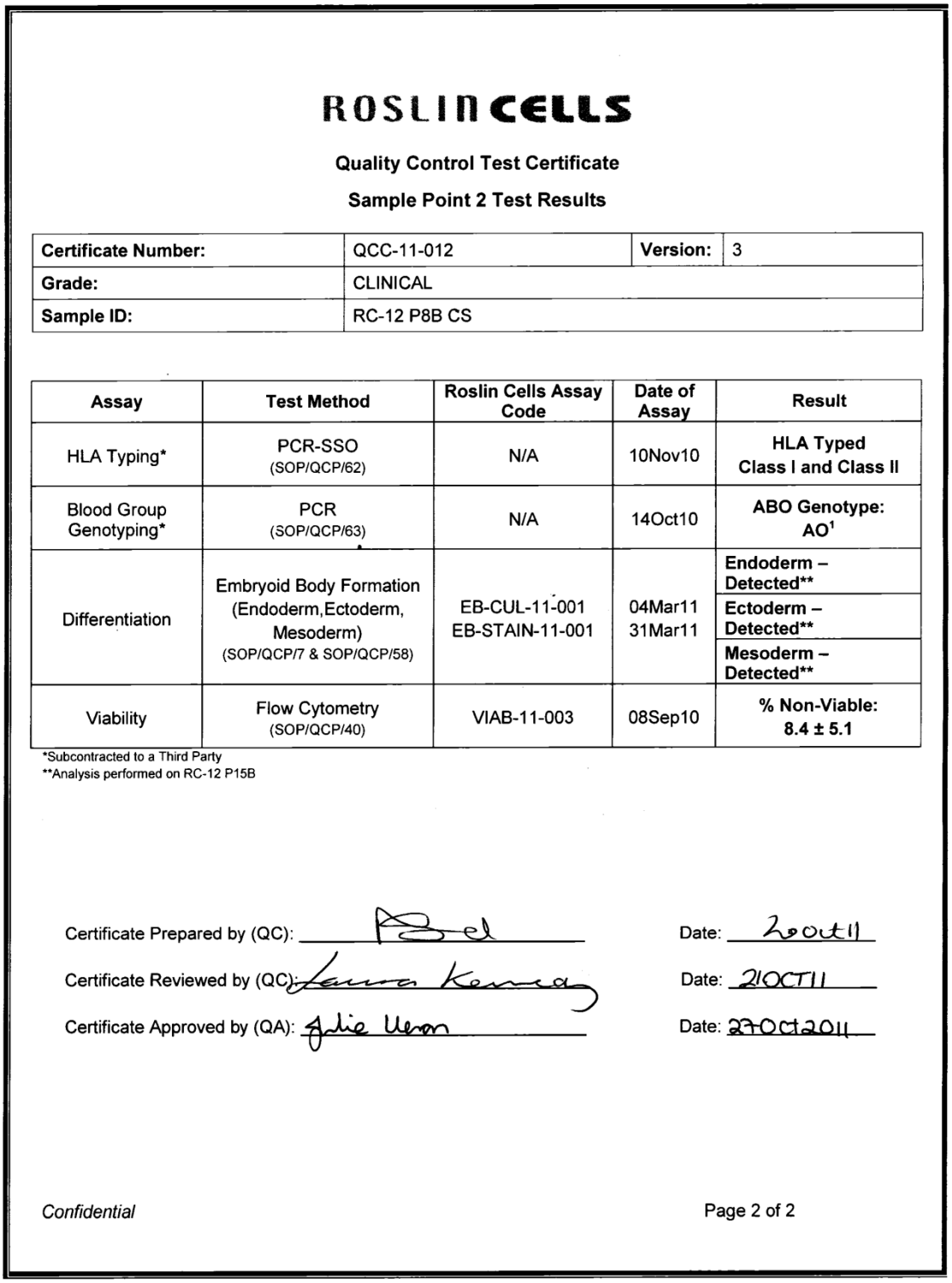
Assay	Test Method	Roslin Cells Assay Code	Date of Assay	Result
Mycoplasma Detection	RT-qPCR (SOP/QCP/22)	MYCO-10-004	07Sep10	**Not Detected*** **
Endotoxin Detection	Kinetic Chromogenic LAL (SOP/QCP/12)	ENDO-10-004	07Sep10	**1.05 EU/ml**
Viral Screening*	PCR (CMV,HTLV1,HIV1,HCV, HBV,EBV) (SOP/QCP/60)	N/A	09Mar11	**Not Detected****
Karyotype*	G-banding (SOP/QCP/59)	N/A	22Mar11	**47,XY,+12(12) /46,XY(18)****
Pluripotency / Differentiation	Flow Cytometry (SOP/QCP/25)	FLOW-10-005	08Sep10	**% Positive** **SSEA-4 – 95.0** **Oct 3/4 – 88.8** **Tra-1-60 – 85.8** **SSEA-1 – 0**
Microsatellite Genotyping*	PCR (SOP/QCP/6)	N/A	14Oct10	**ID Obtained**

*Subcontracted to a Third Party
**Analysis performed on RC-12 P15B
***Refer to IR/0214

Certificate Prepared by (QC): _____ Date: 20Oct11

Certificate Reviewed by (QC): Jamie Kennedy Date: 21OCT11

Confidential



ROSLIN CELLS

Quality Control Test Certificate

Sample Point 2 Test Results

Certificate Number:	QCC-11-012	**Version:**	3
Grade:	CLINICAL		
Sample ID:	RC-12 P8B CS		

Assay	Test Method	Roslin Cells Assay Code	Date of Assay	Result
HLA Typing*	PCR-SSO (SOP/QCP/62)	N/A	10Nov10	**HLA Typed Class I and Class II**
Blood Group Genotyping*	PCR (SOP/QCP/63)	N/A	14Oct10	**ABO Genotype: AO[1]**
Differentiation	Embryoid Body Formation (Endoderm, Ectoderm, Mesoderm) (SOP/QCP/7 & SOP/QCP/58)	EB-CUL-11-001 EB-STAIN-11-001	04Mar11 31Mar11	**Endoderm – Detected****
				Ectoderm – Detected**
				Mesoderm – Detected**
Viability	Flow Cytometry (SOP/QCP/40)	VIAB-11-003	08Sep10	**% Non-Viable: 8.4 ± 5.1**

*Subcontracted to a Third Party
**Analysis performed on RC-12 P15B

Certificate Prepared by (QC): _____ Date: 20 oct 11

Certificate Reviewed by (QC): _____ Date: 21OCT11

Certificate Approved by (QA): _____ Date: 27 Oct 2011

Confidential

Page 2 of 2

Fig. 1. Quality Control Certificate of Analysis for RCe016-A (RC-12) P8B seed lot.

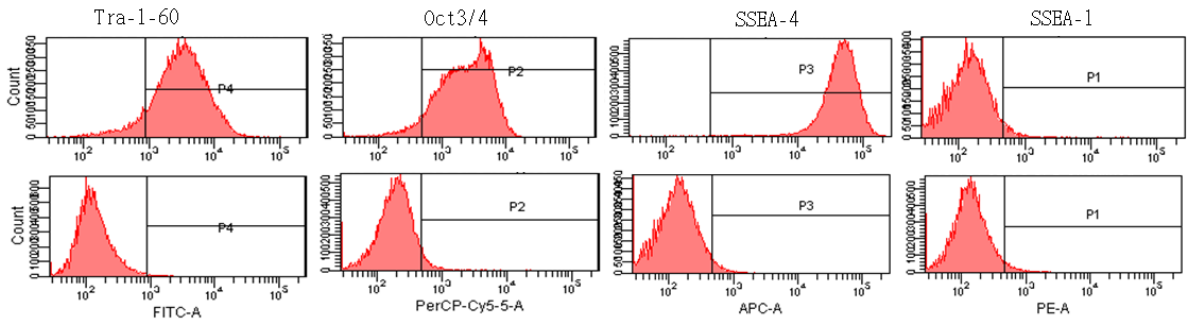


Fig. 2. RCe016-A (RC-12) was subjected to flow cytometry analysis for markers of pluripotency with specific antibody (top row) or isotype control (bottom row) as indicated above the histograms. Percentage staining is indicated in the Certificate of Analysis (Fig. 1).

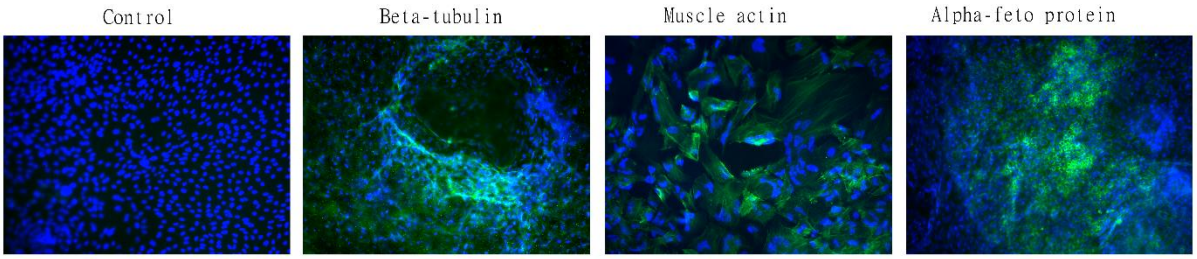


Fig. 3. In vitro differentiation of RCe016-A (RC-12) to ectoderm (β-tubulin III), mesoderm (muscle Actin), and endoderm (α-fetoprotein). Specific staining shown in green, cell nuclei are counterstained with DAPI (blue).

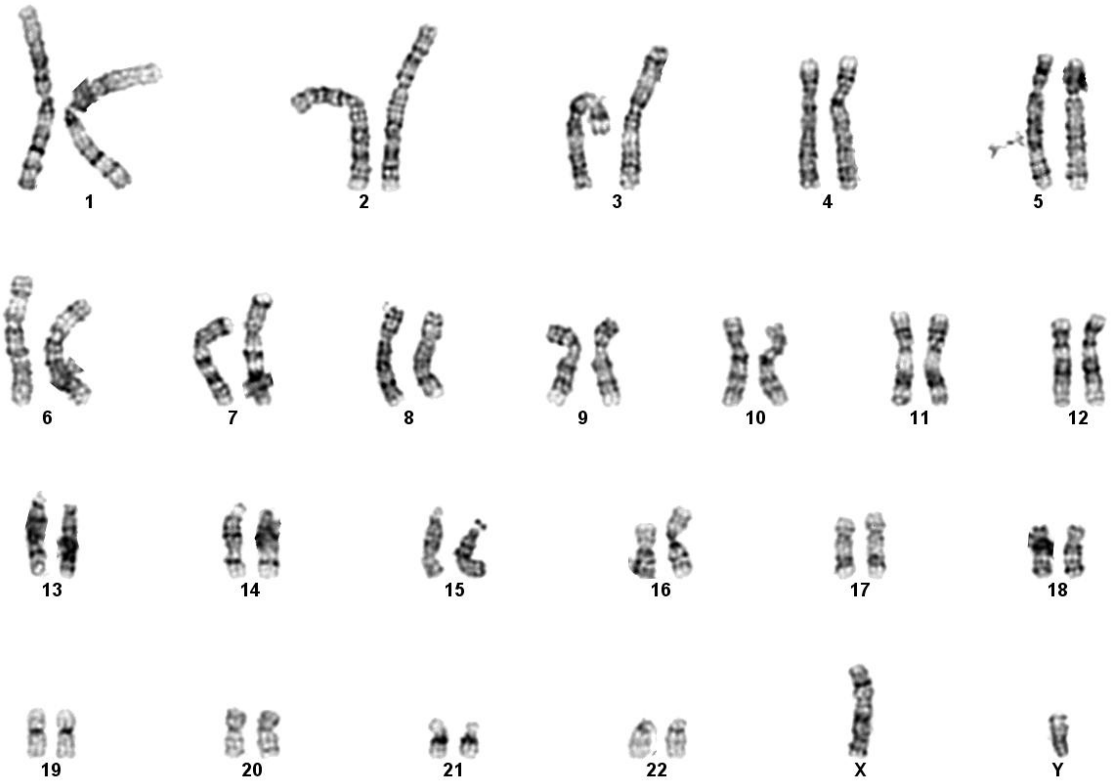


Fig. 4. RCe016-A (RC-12) was analysed by Giesma staining of 20 metaphase spreads at passage 33 and showed a normal male karyotype of 46XY.

Table 1. Microsatellite PCR, blood group and HLA tissue typing results for RCe016-A (RC-12).

Microsatellite PCR results							
D3S1358 1	D3S1358 2	vWA 1	vWA 2	D16S539 1	D16S539 2	D2S1338 1	D2S1338 2
16	17	17	18	9	11	24	25
Amelogenin 1	Amelogenin 2	D8S1179 1	D8S1179 2	D21S11 1	D21S11 2	D18S51 1	D18S51 2
X	Y	13	13	27	29	11	14
D19S433 1	D19S433 2	THO1 1	THO1 2	FGA 1	FGA 2	CSF1PO 1	CSF1PO 2
14	14	7	8	23	26	12	12
D5S818 1	D5S818 2	D7S820 1	D7S820 2	D13S317 1	D13S317 2	TPOX 1	TPOX 2
12	13	11	11	11	13	8	11
Blood group genotyping							
RhD	RhC	Rhc	RhE	Rhe	Fy a	Fy b	Fy GATA
pos	neg	pos	neg	pos	pos	pos	neg
Jka	Jkb	K	k	M	N	S	S
neg	pos	neg	pos	pos	neg	neg	pos

Do a	Do b	ABO
neg	pos	AO1

HLA tissue typing	
HLA Class I Type	HLA-A*01, A*11; B*08, B*35; Cw*04, Cw*07
HLA Class II Type	HLA-DRB1*03, DRB1*04; DRB3*01; DRB4*01; DQB1*02, DQB1*03; DPB1*04
Comment	DRB1*03 is expressed serologically as DR17, DQB1*03 is expressed serologically as DQ8, Unable to exclude DPB1*2302N/2402/9901.

Acknowledgements

Research culminating in the derivation of this line was funded by a grant (PM07321) from Scottish Enterprise Economic Development Agency to PDS, MB, and AC.